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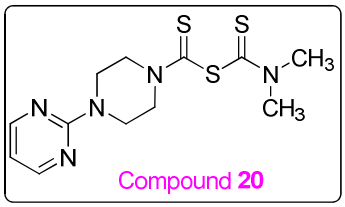
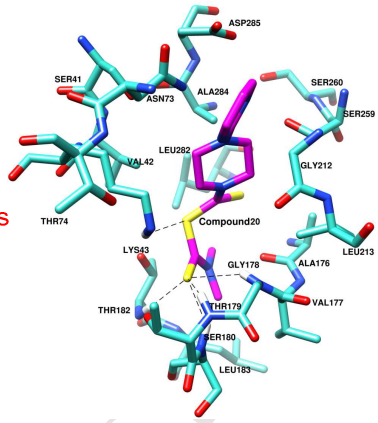


Compound **20**

-MTZ susceptible strain MIC: 4.77μM
-MTZ resistant strain MIC: 38.22μM
-Moderate activity against fungal strains
-Pf3D7(CQ sensitive) IC_{50}: 0.76μM
-PfK1(CQ resistant) IC_{50}: 1.85μM
-Thiol inhibiting agent



Docked complex of most active compound **20** found in modelled cysteine synthase complex

Substituted carbamothioic amine-1-carbothioic thioanhydrides as novel trichomonicidal fungicides: Design, synthesis, and Biology[#]

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Abstract:

Sexually transmitted diseases like trichomoniasis along with opportunistic fungal infections like candidiasis are major global health burden in female reproductive health. In this context a novel non-nitroimidazole class of substituted carbamothioic amine-1-carbothioic thioanhydride series was designed, synthesized, evaluated for trichomonacidal and fungicidal activities, and was found to be more active than the standard drug Metronidazole (MTZ). Compounds were trichomonicidal in the MIC ranges of 4.77–294.1 µM and 32.46–735.20 µM against MTZ-susceptible and -resistant strains, respectively. Further, compounds inhibited the growth of at least two out of ten fungal strains tested at MIC of 7.50-240.38 µM. The most active compound (**20**) of this series was 3.8 and 9.5 fold more active than the MTZ against the two *Trichomonas* strains tested. Compound **20** also significantly inhibited the sulfhydryl groups present over *Trichomonas vaginalis* and was found to be more active than the MTZ *in vivo*. Further, a docking analysis carried out with cysteine proteases supported their thiol inhibiting ability and preliminary pharmacokinetic study has shown good distribution and systemic clearance.

Keywords: Carbamothioic, Thioanhydrides, Trichomoniasis, Candidiasis, Thiol inhibition.

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1. Introduction:

Sexually transmitted infections (STIs) have a major impact on reproductive health of millions of people worldwide.[1, 2] Among the 30 infections identified to be sexually transmitted, incidence and prevalence rate of trichomoniasis is higher than the remaining.[3] Trichomoniasis is a very common, curable, non-viral sexually transmitted urogenital infection in humans caused by the anaerobic, microaerophilic parasite *Trichomonas vaginalis* (TV).[4] Most of the cases in United States and India occur among women of reproductive age[5] and a recent World Health Organization (WHO) report estimates that more than 276 million people are afflicted with trichomoniasis every year.[6] Women and men both can be affected but in men the symptoms are mild and mostly go unreported, which results in its horizontal spread through heterosexual contacts to sexual partners.[2] In men, the observed complications include urethral discharge, dysuria, prostatitis, epididymitis, infertility and benign prostatic hyperplasia.[7-9] In women it is often called “nuisance infection” and may cause endometritis, pyosalpinx, vaginitis, adnexitis, preterm delivery, infertility, bacterial vaginosis and threat of cervical cancer.[10-13] However, TV infections increase the risk of HIV and candidiasis transmissions through colonization of the human urogenital tract epithelium.[14] In sexually active women opportunistic vulvovaginal candidiasis, caused by *Candida albicans* has a high prevalence rate which often co-exists along with TV.[15] The US-FDA approved drug Metronidazole (MTZ), a 5-nitroimidazole, has limited efficacy against drug-resistant Trichomoniasis infections. For decades, the use of MTZ has been notable for its effectiveness but the increasing drug-resistance and cross-resistance to MTZ and other nitroimidazoles (NIs) has been limiting their application in recent times.[16, 17] In 21st century the incidence of drug resistance cases augmented by 17 fold which are being treated typically with increased and probably toxic doses of MTZ.[18, 19] Prolonged treatment or high doses of MTZ may cause side effects such as headache, dry mouth, metallic taste, glossitis and urticaria.[20] Since women are at the receiving end with long term consequences of STIs, lack of effective clinical candidates necessitates the researchers to discover highly effective molecules, preferably non-nitroimidazoles to treat TV and associated infections.[21] Reactive oxygen species (ROS-) sensitive anaerobes TV and *Candida albicans* largely depend on cysteine to overcome redox stress [22] making these infections very susceptible to thiol-inhibiting agents which signifies thiol as vulnerable target for antitrichomonal and antifungal agents.

Our previous research work has established that thiol modifying molecules (dithiocarbamates; **a-d**) are effective against TV and fungal infections. [23-29] Dithiocarbamate (DTC) incorporated MTZ hybrids (**c**) resulted with increased trichomonacidal and spermicidal activities than MTZ.[30] The possibility of drug resistance to nitroimidazole class of compounds necessitates the discovery of non-nitroimidazole compounds. Recently, disulfiram like compounds with two DTCs linked together directly at sulfur atom (**a**) also yielded potent topical dual function microbicides.[31] However like Disulfiram, (which is a drug with two DTC groups and is in clinical use for more than 50 years [32]), these compounds underwent rapid cleavage of S-S bond and failed to show up in pharmacokinetic evaluation after vaginal administration. Since pharmacokinetic evaluation is a mandatory step in modern drug development, it was envisaged that modification of S-S bond to -S- bond which might lead to the discovery of a new class of compounds with effective anti-*Trichomonas* and antifungal activities, and with suitable stability for pharmacokinetic evaluation.

In this present study we evaluated the new compounds against MTZ-susceptible and -resistant TV infections and some fungi that infect the female vaginal tract. A novel series of molecules with improved efficacy over the previous drug designs is being reported along with their *in vivo* biological activities, fluorescence labelling of biological targets and safety evaluation against human epithelium cells (*HeLa*).

(**Figure 1**)

2. Results and Discussion:

2.1 Chemistry

The general procedure for the synthesis of substituted carbamothioic amine-1-carbothioic thioanhydrides (**3**-**21**, **25**-**29** and **33**-**38**) has been depicted in scheme 1. Initially the intermediates sodium substituted amine-1-carbodithioates (**2a**-**2s**, **24a**-**24e** and **31a**-**31f**) were synthesized by the earlier reported methods.[28, 33] In general, various secondary amines (**1a**-**1s**) on reaction with carbon disulfide and aq. sodium hydroxide yield corresponding carbodithioates (**2a**-**2s**).

(Scheme 1)

Further sodium 4-(*tert*-butoxycarbonyl)piperazine-1-carbodithioate on reaction with different alkyl halides gave *tert*-butyl 4-(substitutedthiocarbonothioyl)piperazine-1-carboxylate (**22a**-**22e**). Furthermore Boc removal followed by reaction with carbon disulfide and aq. NaOH yielded sodium 4-(substituted thiocarbonothioyl)piperazine-1-carbodithioates (**24a**-**24e**). Piperazine on reaction with different substituted isothiocyanates/isocyanates in ethanol yielded different urea/thiourea derivatives (**31a**-**31f**) which on reaction with carbon disulfide and aq. NaOH gave sodium 4-(substituted carbomothioyl)piperazine-1-carbodithioates (**32a**-**32f**). Further intermediate compounds **2a**-**2s**, **24a**-**24e** and **31a**-**31f** were on reaction with dimethylcarbamothioic chloride in acetonitrile at 0 ^{o}C for 1-2 hr yielded the final desired products (**3**-**21**, **25**-**29** and **33**-**38**) in good yields.

2.2 Biological Evaluation

2.2.1 Anti-Trichomonas activity of synthesized compounds

All the synthesized compounds (**3**-**21**, **25**-**29** and **33**-**38**) were evaluated for their anti-*Trichomonas* activity against MTZ-susceptible, -resistant strains and safety against cervico epithelium cells (*HeLa*). All the compounds were found to be active at MIC range of 4.77–294.11 µM and 32.46–735.20 µM against MTZ-susceptible and -resistant strains, respectively, where MTZ showed activity at MIC 18.24 and 365.40 µM. Among these four compounds (**12**, **20**, **21** and **34**) were 1 to 3.8 fold more active against -susceptible strain while twenty-six compounds (**3**-**5**, **7**-**15**, **17**-**21**, **26**, **28**, **29**, and **33**-**38**) were 1.3 to 11.2 fold more active against -resistant strain than the standard MTZ. The most active compound (**20**) was 3.8 and 9.5 fold more active than the MTZ against two strains tested. It was also interesting to note that while the standard drug MTZ was twenty folds less active in resistant strain than in susceptible, compound **20** was only 8-fold less active. This could be attributed to the entirely new drug design.

Structure activity relationship (SAR) study: The scaffold studied was substituted carbamothioic amine-1-carbothioic thioanhydrides (**3**-**21**, **25**-**29** and **33**-**38**), which contained four prototypes by varying substituents at -NR^1R^2. Firstly, a single nitrogen amines with cyclic (**3**-**7)** and acyclic (**8**-**10**) framework were synthesized and then a two nitrogen system i.e., substituted piperazines (**11**-**21**) were incorporated at -NR^1R^2. Thereafter a dithiocarbamate (**25**-**29**) and a thiourea (**33**-**38**)

group were integrated at N^4-position of piperazine core. The anti-*Trichomonas* activity data revealed that amine substituents -NR^1R^2 played vital role for the biological activity.

(Table 1) (Figure 2)

Cyclic (**3-7)** and acyclic (**8-10**) amines at -NR^1R^2 showed good to moderate activity (MIC 25.20-112.61 µM) against -susceptible strain while against -resistant strain all the compounds except compound **6** showed 1.5 to 3.8 fold better activity than MTZ irrespective of cyclic or acyclic amines. The replacement of single nitrogen amines by a two nitrogen containing piperazines the compounds exhibited better activity profile. Of the eleven compounds (**11**-**21**) four compounds were comparable (**12**, **13** and **21**) or more active (**20**) than MTZ at MIC 4.77-20.42 µM. The activity data suggested that a 2-pyrimidyl (**20**) substituent at N^4-position was more desirable over alkyl (**11**, **13**)/aryl (**15**-**19**)/carboxy (**12**, **14**)/furoyl (**21**) substitutents. Against –resistant strain except compound **16** remaining ten compounds were 1.3 to 9.5 times more active than MTZ. Here again 2-pyrimidyl (**20**) substituent at N^4-position was essential for resistant anti-*Tichomonas* activity. The order of activity with substituents -NR^1R^2 was 2-pyrimidyl (**20**)>Boc (**12**)>phenyl (**15**)> n-butyl (**13**)>methyl (**11**)>3-CF_3 phenyl (**17**)>2,3-dichlorophenyl (**19**)>furoyl (**21**)>ethoxycarbonyl (**14**)>3-cholorophenyl (**18**)>2-methoxyphenyl (**16**). In the scaffold carbamothioic amine-1-carbothioic thioanhydride attempts were made to hybridize active pharmacophores, dithiocarbamate (**25**-**29**) and thiourea (**33**-**37**) moieties at N^4-position of piperazine. Against MTZ-susceptible strain the incorporation of thiourea moiety was more beneficial over dithiocarbamate as most active compound i.e., with phenyl thiourea (38, MIC 8.10 µM) at N^4-position was 2.2 fold more active and rest of the compounds were less active than standard MTZ. Again the same pattern of activity was observed with resistant strain as compound **34** was 11.4 fold more active than MTZ. The order of activity among dithiocarbamate containing compounds was benzyl (**29**)>butyronitrile (**28**)>ethyl (**26**)>butyl (**27**)>methyl (**25**) while in thiourea derivatives it was phenyl (**34**)>cyclohexyl (**37**)>butyl (**33**)>phenethyl (**36**)>benzyl (**35**). Furthermore a modification of thiourea moiety with urea moiety (**38**) resulted in loss of activity in both the strains tested suggests it least favorable for desired activity.

2.2.2 Antifungal activity of synthesized compounds

All the synthesized compounds (**3-21**, **25-29** and **33-38**) were also evaluated for antifungal activity against ten fungal strains including three opportunistic fungal strains and seven *Candida* strains. The activity data showed that twelve compounds (**3-7**, **9-13**, **20** and **21**) were active against all the ten fungal strains while remaining compounds inhibited the growth of at least two fungal strains tested in the range of 7.50-240.38 µM. The amine substituents at -NR^1R^2 played a significant role for antifungal activity as like in case of anti-*Trichomonas* activity. Substitutions at -NR^1R^2 with cyclic/acyclic secondary amines (**3-11**) were found to be active at MIC 11.90-240.38 µM where various substituted piperazine compounds (**12-21**) showed activity in a range of MIC 8.66-163.39 µM. Further a DTC incorporated piperazine compounds (**25-29**) have shown activity at MIC 7.50-147.05 µM while thiourea/urea hybridized compounds (**33-38**) exhibited fungicidal activity at MIC 125.31-136.98 µM except compound **36** which was inactive in all the ten strains tested. These fungicidal results suggested that in the prototype where DTC was incorporated in piperazine core at -NR^1R^2 was more favorable over other three prototypes. It was interesting to note that except one compound (**36**), all the remaining twenty nine compounds were found to be active against at least two opportunistic fungal starins; *cryptococcus neoformans*, *sporothrix schenckii*, *trichophyton mentagrophytes* at MIC 15.86-240.38 µM. One compound **7** against *Candida glabrata* and four compounds (**18**, **26**, **27** and **29**) against *Candida parapsilosis* had activity comparable to standard Flucanazole. Many volvovaginal infections were associated with *Candida* infections which coheres to vaginal epithelium cells enhances the transmission of STIs. Among compounds evaluated, twelve (**3-7**, **9-13**, **20** and **21**) have shown activity against all the *candida* strains tested. Rest of the compounds except compounds **28** and **34-36** were active against at least one out of seven different *candida* strains evaluated.

(**Table 2**)

Since the compounds exhibited good activity against the protozoan STD-pathogen (*T. vaginalis*). We also tested some of the promising structures (**12**, **20**, **21**, **34**) against *P. falciparum* to observe if the effects are universal to all kinds of protozoa or specific only to *Trichomonas*.

These compounds were less active than standard antimalarial chloroquine (Supplementary Data Table 1).

*2.2.3 Thiol Inhibition by Active Compound **20:***

The lack of glutathione makes *T. vaginalis* to depend on cysteine to overcome redox stress, making very susceptible to sulfhydryl manipulating agents.[22] The DTC compounds were known to be sulfhydryl binding agents. With the presence of DTC in the most active compound **20** it was evaluated for its sulfhydryl binding ability. The inhibition of sulfhydryl groups over *Trichomonas vaginalis* were localized qualitatively by fluorescence detection after labeling them with mBBr dye. Motile *Trichomonas vaginalis* (control) and compound **20** treated *Trichomonas* were digitally imaged for qualitative assessment. From figure 2, a significant inhibition of free thiol fluorescence was observed with compound **20** treatment. In control group due to the presence of higher number of free sulfhydryl groups the fluorescence intensities was higher than in compound **20** treated groups. The decreased fluorescence indicates the interaction of compound **20** with the sulfhydryls present over *Trichomonas*, which might be its trichomonacidal mechanism.

(**Figure 3**)

2.2.4 Docking study

Further a docking study was also carried out with most active compound (**20**) to ascertain their cysteine biosynthesis pathway. Cysteine is vital for all living life forms for protein synthesis, as a precursor for glutathione and biomolecules also as a source for synthesis of iron-sulfur clusters. This series of compounds possibly exhibit anti-*Trichomonas* activity by inhibiting the cysteine biosynthesis pathway. To acquire knowledge about the binding mode of the most promising compound **20** found in this study, we have performed the docking study with the homology model of Cysteine Synthase (CS). The validation of the resulting model was done with the Structural Analysis and Verification Server (SAVS) [42]. In the selected model the majority of the residues (97%) occupy the most favourable region of Ramachandran plot and 2% and 1% residue lie in additionally allowed region and generously allowed region [31]. This model was used for docking studies. The Pyridoxal-5'-phosphate (PLP) binding site was taken for the

docking of the most active compound. It was observed that compound **20** docked well in this cavity with binding energy of -8.40 kcal/mol. The binding mode of docked complex is shown in Figure 4. It was found that compound **20** is involved in forming a hydrogen bond with catalytically important residue Lys43, which is supposed to interact covalently with PLP. The docked complex was further stabilized by interactions with residues Gly178, Thr182, Thr179 and Ser180 present in the active site.

(**Figure 4**)

*2.2.5 In vivo anti-Trichomonas activity of compound **20**:*

Due to its better *in vitro* activity and safety against *HeLa* cells, compound **20** was further screened for *in vivo* anti-*Trichomonas* activity. The *in vivo* efficacy of compound was evaluated using the mouse abscess assay. Subcutaneous injection of live trichomonads resulted in a small pustule of ~50 mm^2 area on day-7 of injection (day-1 of treatment) in experimental and control animals that grew to ~96.55 mm^2 in area in controls but was reduced to ~1-2.5 mm^2 after 5-days of treatment with compound and MTZ (Figure 4). Thereafter the growth of abscess was exponential in controls and it was >100 mm^2 in area after 7 days. However, it was reduced to ~0.8–2.5 mm^2 in compound-**20** treated animals. On the day of autopsy (i.e. the day following seven days of treatment), the abscess area measured ~96.5 mm^2 in control animals and 2.35 mm^2 in 50 mg of MTZ, 1.57 and 0.79 mm^2 in 50 mg and 100 mg of compound-**20** treated animals, respectively.

(Figure 5)

2.2.6 Pharmacokinetics study

A preliminary pharmacokinetic study of compound **20** involved monitoring drug substance in blood plasma. The animals tolerated the treatment well as no peculiarities in the animals' behaviour were observed. Following 50 mg/kg oral dose of compound **20,** peak serum concentration C_{max}= 47.1±2.8 ng/mL was achieved at 2 hr and could be monitored in serum up to 6 hr post dose. The concentration-time profile was analyzed using non-compartmental approach and the calculated pharmacokinetic parameters are shown in Table 3. The pharmacokinetic models were compared according to maximal correlation between observed and predicted

concentration, minimal sum of squared residuals, Akaike's Information Criterion (AIC) and Schwarz Bayesian Criterion (SBC) [34, 35]. The volume of distribution (12.6±0.6 L/kg) was larger than the total blood volume of rat (0.054 L/kg; [36]) and systemic clearance (4.9±0.2 L/h/kg) was higher than the total hepatic blood flow in rats (2.9 L/h/kg; [36]) indicating extravascular distribution along with the extra hepatic elimination of the compound.

(Table 3)

3. Conclusion

Sexually transmitted diseases like trichomoniasis along with opportunistic fungal infections like candidiasis are major global concern in female reproductive health now days. The increased drug resistance to available 5-nitroimidazole drugs i.e., Metronidazole, Tinidazole necessitates the researchers for new drug development. Developing non-nitro imidazole molecules to overcome the drug resistance would be a useful strategy. In the present study we developed a novel series of non-nitroimidazole molecules and evaluated their trichomonicidal and fungicidal efficacies. All the compounds were found to be trichomonicidal at MIC range of 4.77–294.11 µM and 32.46–735.20 µM against MTZ-susceptible and -resistant strains, respectively. MTZ showed activity at MIC of 18.24 and 365.40 µM respectively against the strains. Four compounds (**12**, **20**, **21** and **34**) were up to 3.8 fold more active against -susceptible strain and twenty-six compounds (**3-5**, **7-15**, **17-21**, **26**, **28**, **29** and **33-38**) were up to 11.2 fold more active against resistant strain than the standard MTZ. The most active compound (**20**) was 3.8 and 9.5 fold more active than MTZ against susceptible and resistant strains, respectively. The absence of nitro group in this novel series of compounds could be the reason for the activity of compounds against resistant trichomoniasis. Twenty-nine compounds were found to be active against at least two opportunistic fungal strains at MIC 15.86-240.38 µM. Many vulvovaginal infections are associated with *Candida* infections, which cohere to vaginal epithelium cells and enhance the transmission of STIs. Among the compounds evaluated twelve (**3-7**, **9-13**, **20** and **21**) have shown activity against all the ten *candida* strains tested at MIC 7.50-200.00 µM. Compound **20** significantly inhibited the free sulfhydryl groups presented over *Trichomonas in vitro*, and demonstrated significant elimination of infection in *in vivo* assay at doses 50 mg/kg and 100 mg/kg compound. Most active compounds also have shown moderate antimalarial activity. A

preliminary pharmacokinetic study has shown good distribution, systemic clearance of compound **20** in SD-rats. The present study has identified promising structures that could conquer the drug-resistance in Trichomonas and their further lead optimization may lead to the identification of more effective non-nitro imidazole class of anti-microbial compounds.

Experimental Section

Chemistry

In general, all reagents and solvents were of commercial quality and were used without further purification. All reactions were monitored by thin-layer chromatography (TLC) using F_{254} silica gel plates with fluorescence (Aldrich). Melting points were determined in open capillary tubes on an electrically heated block and are uncorrected. IR spectra (ν_{max} in cm^{-1}) of the compounds were recorded on Perkin Elmer FT-IR RX1 PC spectrophotometer. ^{1}H NMR spectra were recorded on Bruker Supercon Magnet Avance DPX-200/DRX-300 spectrometers (operating at 400 and 100 MHz, respectively, for ^{1}H and ^{13}C) in deuterated solvents with TMS as internal reference (chemical shifts δ in ppm, *J* in Hz.). Electrospray ionization mass spectra (ESI-MS) were recorded on Ion Trap LCQ Advantage Max-IT (Thermo Electron Corporation). High-resolution mass spectra (HRMS) were recorded on a 6520 Agilent Q Tof LC MS/MS (accurate mass). Elemental analyses were performed on a Carlo Erba EA-1108 micro analyzer/Vario EL-III C, H, N analyzer. All compounds were analyzed of C, H, N and the results obtained were within ± 0.4% of calculated values. All final compounds were found to have >95% purity.

Intermediate compounds **2a-2s**, **24a-24e** and **31a-31f** Compounds were synthesized according to a previously reported procedure.[28, 33]

General procedure for the synthesis of dimethylcarbamothioic pyrrolidine-1-carbothioic thioanhydride (3):

To a solution of sodium pyrrolidine-1-carbodithioate (**2a**; 2 equiv.) in 10 mL acetonitrile at 0-5 ^{o}C was added dimethylcarbamothioic chloride (1 equiv.) and stirred the contents for 1-2 hr. After completion of reaction (as monitored by TLC) acetonitrile was

evaporated and excess distilled water (3 x 30 mL), 20 mL of EtOAc were added to the reaction mixture. Organic layer was separated, dried (over Na_2SO_4) and concentrated under reduced pressure to get the title compound **3** in 79% yields as yellow solid. mp: 79-81 °C; IR (KBr) ν (cm^{-1}): 3019, 1218; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.89 (t, J = 7.0 Hz, 2H), 3.73 (t, J = 6.8 Hz, 2H), 3.54 (s, 3H), 3.47 (s, 3H), 2.12–2.00 (m, 4H); ^{13}C (100 MHz, $CDCl_3$): δ 186.9, 183.2, 55.0, 52.8, 45.0, 44.3, 26.3, 24.7; ESI-MS: *m/z* 235 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_8H_{14}N_2S_3$ + H^+ ($M+H^+$): 235.0392. Found: 235.0389. Elemental analysis (%) for $C_8H_{14}N_2S_3$: Calcd.: C, 40.99; H, 6.02; N, 11.95; Found, C, 40.63; H, 6.38; N, 11.79.

The compounds **4-21**, **25-29** and **33-38** were synthesized by using a procedure similar to described for compound **3**.

Dimethylcarbamothioic piperidine-1-carbothioic thioanhydride (4)

The title compound (**4**) was synthesized from sodium piperidine-1-carbodithioate (**2b**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 71% yield as yellow solid. mp: 90-92 °C; IR (KBr) ν (cm^{-1}): 3019, 1214; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02 (s, 1H), 4.26 (bs, 2H), 3.89 (bs, 2H), 3.53 (s, 3H), 3.44 (s, 3H), 1.74–1.61 (m, 6H); ^{13}C (100 MHz, $CDCl_3$): δ 187.6, 185.7, 54.0, 52.2, 44.9, 44.0, 26.3, 25.2, 23.8; ESI-MS: *m/z* 249 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_9H_{16}N_2S_3$ + H^+ ($M+H^+$): 249.0548. Found: 249.0549. Elemental analysis (%) for $C_9H_{16}N_2S_3$: Calcd.: C, 43.51; H, 6.49; N, 11.28; Found, C, 43.15; H, 6.76; N, 11.39.

Dimethylcarbamothioic 3-methylpiperidine-1-carbothioic thioanhydride (5)

The title compound (**5**) was synthesized from sodium 3-methylpiperidine-1-carbodithioate (**2c**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 82% yield as yellow solid. mp: 95-97 °C; IR (KBr) ν (cm^{-1}): 3021, 1216; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.24 (bs, 1H), 4.42–4.30 (m, 1H), 3.54–3.44 (m, 6H), 3.27–3.19 (m, 1H), 3.01–2.83 (m, 1H), 1.91–1.83 (m, 3H), 1.72–1.65 (m, 2H), 0.98 (bs, 3H); ^{13}C (100 MHz, $CDCl_3$): δ 187.3, 185.8, 59.8, 58.0, 53.5, 51.8, 44.9, 44.0, 32.4, 32.0, 31.1, 25.7, 24.4,

18.7; ESI-MS: *m/z* 263 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_{10}H_{18}N_2S_3$ + H^+ ($M+H^+$): 263.0705. Found: 263.0724. Elemental analysis (%) for $C_{10}H_{18}N_2S_3$: Calcd.: C, 45.76; H, 6.91; N, 10.67; Found, C, 45.99; H, 7.21; N, 10.80.

Dimethylcarbamothioic morpholine-4-carbothioic thioanhydride (6)

The title compound (**6**) was synthesized from sodium morpholine-4-carbodithioate (**2d**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 78% yield as yellow solid. mp: 128-130 °C; IR (KBr) ν (cm^{-1}): 3019, 1215, 1104; ^1H NMR (400 MHz, $CDCl_3$): δ 4.31–3.99 (m, 4H), 3.83 (bs, 4H), 3.52 (s, 3H), 3.42 (s, 3H); ^{13}C (100 MHz, $CDCl_3$): δ 187.5, 186.5, 66.1, 52.9, 50.9, 44.9, 43.6; ESI-MS: *m/z* 251 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_8H_{14}N_2OS_3$ + H^+ ($M+H^+$): 251.0341. Found: 251.0334. Elemental analysis (%) for $C_8H_{14}N_2OS_3$: Calcd.: C, 38.37; H, 5.64; N, 11.19; Found, C, 38.01; H, 5.98; N, 11.32.

Azepane-1-carbothioic dimethylcarbamothioic thioanhydride (7)

The title compound (**7**) was synthesized from sodium azepane-1-carbodithioate (**2e**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 85% yield as yellow solid. mp: 75-77 °C; IR (KBr) ν (cm^{-1}): 3010, 1216; ^1H NMR (400 MHz, $CDCl_3$): δ 4.14 (t, J = 6.0 Hz, 2H), 3.87 (t, J = 6.0 Hz, 2H), 3.54 (s, 3H), 3.47 (s, 3H), 1.91–1.84 (m, 4H), 1.64–1.61 (m, 4H); ^{13}C (100 MHz, $CDCl_3$): δ 187.2, 187.0, 55.0, 54.8, 45.0, 44.5, 28.0, 26.9, 26.4, 25.8; ESI-MS: *m/z* 263 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_{10}H_{18}N_2S_3$ + H^+ ($M+H^+$): 263.0705. Found: 263.0702. Elemental analysis (%) for $C_{10}H_{18}N_2S_3$: Calcd.: C, 45.76; H, 6.91; N, 10.67; Found, C, 45.45; H, 7.22; N, 10.49.

Diethyl-1-carbothioic dimethylcarbamothioic thioanhydride (8)

The title compound (**8**) was synthesized from sodium diethylcarbamodithioate (**2f**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 70% yield as yellow solid. mp: 84-86 °C; IR (KBr) ν (cm^{-1}): 3020, 1211; ^1H NMR (400 MHz, $CDCl_3$): δ 4.02–3.97 (m, 2H), 3.76–3.68 (m, 2H), 3.53 (s, 3H), 3.45 (s, 3H), 1.35–1.29 (m, 6H); ^{13}C (100 MHz, $CDCl_3$): δ 187.1, 186.5, 49.0, 48.9, 45.0, 44.4, 13.2, 11.1; ESI-MS: *m/z* 237 ($M+H^+$);

Elemental analysis (%) for $C_8H_{16}N_2S_{32}$: Calcd.: C, 40.64; H, 6.82; N, 11.85; Found, C, 40.50; H, 6.99; N, 11.61.

Dimethyl-1-carbothioic thioanhydride (9)

The title compound (**9**) was synthesized from sodium dimethylcarbamodithioate (**2g**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 68% yield as yellow solid. mp: 89-91 °C; IR (KBr) ν (cm^{-1}): 3019, 1218; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.53 (s, 6H), 3.44 (s, 6H); ^{13}C (100 MHz, $CDCl_3$): δ 187.3, 44.9, 44.0; ESI-MS: *m/z* 209 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_6H_{12}N_2S_3 + H^+$ ($M+H^+$): 209.0241. Found: 209.0282. Elemental analysis (%) for $C_6H_{12}N_2S_3$: Calcd.: C, 34.59; H, 5.80; N, 13.44; Found, C, 34.37; H, 5.99; N, 13.56.

Dimethylcarbamothioic ethyl(methyl)-4-carbothioic thioanhydride (10)

The title compound (**10**) was synthesized from sodium ethyl(methyl)carbamodithioate (**2h**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 84% yield as yellow solid. mp: 85-87 °C; IR (KBr) ν (cm^{-1}): 3010, 1210; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.12–4.03 (m, 1H), 3.82–3.77 (m, 1H), 3.55–3.37 (m, 9H), 1.31 (t, *J* = 7.1 Hz, 3H); ^{13}C (100 MHz, $CDCl_3$): δ 187.3, 187.1, 51.8, 51.5, 44.9, 44.0, 42.4, 41.1, 12.9, 10.7; ESI-MS: *m/z* 223 ($M+H^+$); Elemental analysis (%) for $C_7H_{14}N_2S_3$: Calcd.: C, 37.80; H, 6.35; N, 12.60; Found, C, 37.54; H, 6.70; N, 12.84.

Dimethylcarbamothioic 4-methylpiperazine-1-carbothioic thioanhydride (11)

The title compound (**11**) was synthesized from sodium 4-methylpiperazine-1-carbodithioate (**2i**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 88% yield as yellow solid. mp: 70-72 °C; IR (KBr) ν (cm^{-1}): 3011, 1218, 1167; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.34–3.98 (m, 4H), 3.53 (s, 3H), 3.44 (s, 3H), 2.58 (s, 4H), 2.36 (s, 3H); ^{13}C (100 MHz, $CDCl_3$): δ 187.4, 186.7, 54.4, 53.9, 52.2, 50.5, 45.3, 44.9, 43.8; ESI-MS: *m/z* 264 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_9H_{17}N_3S_3 + H^+$ ($M+H^+$): 264.0657. Found: 264.0656. Elemental analysis (%) for $C_9H_{17}N_3S_3$: Calcd.: C, 41.03; H, 6.50; N, 15.95; Found, C, 41.25; H, 6.76; N, 15.59.

4-(*tert*-Butoxycarbonyl)piperazine-1-carbothioic dimethylcarbamothioic thioanhydride (12)

The title compound (**12**) was synthesized from sodium 4-(*tert*-butoxycarbonyl)piperazine-1-carbodithioate (**2j**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 85% yield as yellow solid. mp: 201-203 °C; IR (KBr) ν (cm^{-1}): 3010, 1730, 1216, 1020; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.29–3.97 (m, 4H), 3.64–3.61 (m, 4H), 3.53 (s, 3H), 3.42 (s, 3H), 1.49 (s, 9H); ^{13}C (100 MHz, $CDCl_3$): δ 187.5, 186.7, 154.3, 80.6, 52.1, 50.6, 44.9, 43.6, 42.7, 28.3; ESI-MS: *m/z* 350 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_{13}H_{23}N_3O_2S_3 + H^+$ ($M+H^+$): 350.1025. Found: 350.1029. Elemental analysis (%) for $C_{13}H_{23}N_3O_2S_3$: Calcd.: C, 44.67; H, 6.63; N, 12.02; Found, C, 44.39; H, 6.89; N, 12.15.

4-Butylpiperazine-1-carbothioic dimethylcarbamothioic thioanhydride (13)

The title compound (**13**) was synthesized from sodium 4-butylpiperazine-1-carbodithioate (**2k**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 77% yield as yellow solid. mp: 102-104 °C; IR (KBr) ν (cm^{-1}): 3015, 1215, 1165; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.32–3.96 (m, 4H), 3.53 (s, 3H), 3.43 (s, 3H), 2.59 (bs, 4H), 2.42–2.38 (m, 2H), 1.53–1.45 (m, 2H), 1.40–1.33 (m, 2H), 0.94 (t, J = 7.3 Hz, 3H); ^{13}C (100 MHz, $CDCl_3$): δ 187.4, 186.4, 57.6, 52.7, 52.1, 50.6, 44.9, 43.8, 28.9, 20.5, 13.9; ESI-MS: *m/z* 306 ($M+H^+$); Elemental analysis (%) for $C_{12}H_{23}N_3S_3$: Calcd.: C, 47.17; H, 7.59; N, 13.75; Found, C, 47.00; H, 7.86; N, 13.57.

4-(Ethoxycarbonyl) piperazine-1-carbothioic dimethylcarbamothioic thioanhydride (14)

The title compound (**14**) was synthesized from sodium 4-(ethoxycarbonyl)piperazine-1-carbodithioate (**2l**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 80% yield as yellow solid. mp: 112-114 °C; IR (KBr) ν (cm^{-1}): 3011, 1736, 1216; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.25–3.99 (m, 6H), 3.67–3.64 (m, 4H), 3.50 (s, 3H), 3.40 (s, 3H), 1.27 (t, J = 7.1 Hz, 3H); ^{13}C (100 MHz, $CDCl_3$): δ 187.5, 186.8, 155.5, 61.8, 51.9, 50.6, 44.9,

43.6, 42.9, 14.6; ESI-MS: *m/z* 322 ($M+H^+$); Elemental analysis (%) for $C_{11}H_{19}N_3O_2S_3$: Calcd.: C, 41.10; H, 5.96; N, 13.07; Found, C, 41.00; H, 6.06; N, 13.10.

Dimethylcarbamothioic 4-phenylpiperazine-1-carbothioic thioanhydride (15)

The title compound (**15**) was synthesized from sodium 4-phenylpiperazine-1-carbodithioate (**2m**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 79% yield as yellow solid. mp: 88-90 °C; IR (KBr) ν (cm^{-1}): 3410, 1427, 1215, 1150; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.33–7.29 (m, 2H), 6.95–6.92 (m, 3H), 4.46–4.11 (m, 4H), 3.54 (s, 3H), 3.45 (s, 3H), 3.39–3.37 (m, 4H); ^{13}C (100 MHz, $CDCl_3$): δ 187.5, 186.5, 150.1, 129.3, 120.6, 116.3, 50.6, 49.1, 48.4, 44.9, 43.8; ESI-MS: *m/z* 326 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_{14}H_{19}N_3S_3 + H^+$ ($M+H^+$): 326.0814. Found: 326.0820. Elemental analysis (%) for $C_{14}H_{19}N_3S_3$: Calcd.: C, 51.66; H, 5.88; N, 12.91; Found, C, 51.59; H, 6.11; N, 12.83.

4-(2-Methoxyphenyl)piperazine-1-carbothioic dimethylcarbamothioic thioanhydride (16)

The title compound (**16**) was synthesized from sodium 4-(2-methoxyphenyl)piperazine-1-carbodithioate (**2n**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 81% yield as yellow solid. mp: 170-172 °C; IR (KBr) ν (cm^{-1}): 2925, 1468, 1225, 1026; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.09–7.05 (m, 1H), 6.98–6.91 (m, 3H), 4.51 (bs, 2H), 4.17–4.11 (m, 2H), 3.90 (s, 3H), 3.55–3.46 (m, 6H), 3.23 (s, 4H); ^{13}C (100 MHz, $CDCl_3$): δ 187.5, 186.5, 152.2, 139.9, 123.8, 121.1, 118.5, 111.4, 55.4, 52.8, 50.9, 50.4, 49.8, 44.9, 43.8; ESI-MS: *m/z* 356 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_{15}H_{21}N_3OS_3 + H^+$ ($M+H^+$): 356.0920. Found: 356.0919. Elemental analysis (%) for $C_{15}H_{21}N_3OS_3$: Calcd.: C, 50.67; H, 5.95; N, 11.82; Found, C, 50.50; H, 6.15; N, 11.78.

4-(2-(Trifluoromethyl)phenyl)piperazine-1-carbothioic dimethylcarbamothioic thioanhydride (17)

The title compound (**17**) was synthesized from sodium 4-(2-(trifluoromethyl)phenyl)piperazine-1-carbodithioate (**2o**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 75% yield as yellow solid. mp: 95-97 °C; IR

(KBr) ν (cm^{-1}): 3020, 1429, 1215, 1028; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40 (t, J = 7.9 Hz, 1H), 7.16–7.06 (m, 3H), 4.48–4.16 (m, 4H), 3.54 (s, 3H), 3.46–3.45 (m, 7H); ^{13}C (100 MHz, $CDCl_3$): δ 187.5, 186.6, 150.2, 132.0, 131.7, 131.4, 131.0, 129.8, 125.5, 122.8, 118.8, 116.5, 116.5, 112.1, 112.1, 51.9, 50.4, 48.1, 44.9, 43.7; ESI-MS: *m/z* 394 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_{15}H_{18}F_3N_3S_3 + H^+$ ($M+H^+$): 394.0688. Found: 394.0692. Elemental analysis (%) for $C_{15}H_{18}F_3N_3S_3$: Calcd.: C, 45.78; H, 4.61; N, 10.68; Found, C, 45.47; H, 4.90; N, 10.50.

4-(3-Chlorophenyl)piperazine-1-carbothioic dimethylcarbamothioic thioanhydride (18)

The title compound (**18**) was synthesized from sodium 4-(2-chlorophenyl)piperazine-1-carbodithioate (**2p**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 73% yield as yellow solid. mp: 90-92 °C; IR (KBr) ν (cm^{-1}): 3021, 1215, 1152, 758; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.23–7.19 (m, 1H), 6.90–6.87 (m, 2H), 6.82–6.77 (m, 1H), 4.48–4.43 (m, 2H), 4.18–4.11 (m, 2H), 3.54 (s, 3H), 3.44 (m, 3H), 3.41–3.38 (m, 4H); ^{13}C (100 MHz, $CDCl_3$): δ 187.5, 186.6, 151.0, 135.1, 130.3, 120.1, 115.9, 113.9, 51.1, 48.6, 48.5, 44.9, 43.7; ESI-MS: *m/z* 360 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_{14}H_{18}ClN_3S_3 + H^+$ ($M+H^+$): 360.0424. Found: 360.0424. Elemental analysis (%) for $C_{14}H_{18}ClN_3S_{32}$: Calcd.: C, 46.71; H, 5.04; N, 11.67; Found, C, 46.90; H, 5.37; N, 11.50.

4-(2,3-Dichlorophenyl)piperazine-1-carbothioic dimethylcarbamothioic thioanhydride (19)

The title compound (**19**) was synthesized from sodium 4-(2,3-dichlorophenyl)piperazine-1-carbodithioate (**2q**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 80% yield as yellow solid. mp: 235-237 °C; IR (KBr) ν (cm^{-1}): 3019, 1423, 1215, 757; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.25–7.17 (m, 2H), 6.96 (d/d, J = 1.7 & 7.8 Hz, 1H), 4.52–4.51 (m, 2H), 4.18–4.11 (m, 2H), 3.55 (s, 3H), 3.45 (m, 3H), 3.22–3.20 (m, 4H); ^{13}C (100 MHz, $CDCl_3$): δ 187.5, 186.8, 149.9, 134.2, 127.7, 127.6, 125.5, 118.8, 52.8, 51.0, 50.4, 44.9, 43.7; ESI-MS: *m/z* 394 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_{14}H_{17}Cl_2N_3S_3 + H^+$ ($M+H^+$): 394.0034. Found: 394.0035. Elemental analysis (%) for $C_{14}H_{17}Cl_2N_3S_3$: Calcd.: C, 42.63; H, 4.34; N, 10.65; Found, C, 42.79; H, 4.60; N, 10.39.

Dimethylcarbamothioic 4-(pyrimidin-2-yl)piperazine-1-carbothioic thioanhydride (20)

The title compound (**20**) was synthesized from sodium 4-(2,3-dichlorophenyl)piperazine-1-carbodithioate (**2r**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 84% yield as yellow solid. mp: 180-182 °C; IR (KBr) ν (cm^{-1}): 3019, 1500, 1427, 1215; ^1H NMR (400 MHz, $CDCl_3$): δ 8.36 (d, J = 4.7 Hz, 2H), 6.59 (t, J = 4.7 Hz, 1H), 4.39–4.03 (m, 8H), 3.54 (s, 3H), 3.45 (m, 3H); ^{13}C (100 MHz, $CDCl_3$): δ 187.4, 186.6, 161.1, 157.8, 110.7, 52.0, 50.7, 44.9, 43.7, 42.5; ESI-MS: *m/z* 328 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_{12}H_{17}N_5S_3$ + H^+ ($M+H^+$): 328.0719. Found: 328.0718. Elemental analysis (%) for $C_{12}H_{17}N_5S_3$: Calcd.: C, 44.01; H, 5.23; N, 21.38; Found, C, 44.30; H, 5.55; N, 21.09.

4-(furan-2-carbonyl)piperazine-1-carbothioic dimethylcarbamothioic thioanhydride (21)

The title compound (**21**) was synthesized from sodium 4-(furan-2-yl)piperazine-1-carbodithioate (**2s**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 77% yield as yellow solid. mp: 84-86 °C; IR (KBr) ν (cm^{-1}): 3017, 1422, 1216, 1090; ^1H NMR (400 MHz, $CDCl_3$): δ 7.52 (d, J = 0.7 Hz, 1H), 7.13–7.11 (m, 1H), 6.54–6.52 (m, 1H), 4.36–4.03 (m, 8H), 3.53 (s, 3H), 3.43 (m, 3H); ^{13}C (100 MHz, $CDCl_3$): δ 187.6, 186.8, 159.0, 147.4, 144.2, 117.5, 111.6, 51.8, 50.7, 45.6, 44.9, 43.5; ESI-MS: *m/z* 418 ($M+H^+$); Elemental analysis (%) for $C_{13}H_{17}N_3O_2S_3$: Calcd.: C, 45.46; H, 4.99; N, 12.23; Found, C, 45.29; H, 5.33; N, 12.00.

Dimethylcarbamothioic 4-(methylthiocarbonothioyl)piperazine-1-carbothioic thioanhydride (25)

The title compound (**25**) was synthesized from sodium 4-(methylthiocarbonothioyl)piperazine-1-carbodithioate (**24a**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 77% yield as yellow solid. mp: 235-237 °C; IR (KBr) ν (cm^{-1}): 3022, 1215; ^1H NMR (400 MHz, $CDCl_3$): δ 4.35–4.11 (m, 8H), 3.53–

3.42 (m, 6H), 2.70 (s, 3H); ^{13}C (100 MHz, $CDCl_3$): δ 199.0, 187.6, 186.5, 50.4, 48.5, 45.0, 43.5, 20.0; ESI-MS: *m/z* 340 ($M+H^+$); Elemental analysis (%) for $C_{10}H_{17}N_3S_5$: Calcd.: C, 35.37; H, 5.05; N, 12.37; Found, C, 35.09; H, 5.31; N, 12.12.

4-(Ethylthiocarbonothioyl)piperazine-1-carbothioic dimethylcarbamothioic thioanhydride (26)

The title compound (**26**) was synthesized from sodium 4-(ethylthiocarbonothioyl)piperazine-1-carbodithioate (**24b**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 70% yield as yellow solid. mp: 98-100 °C; IR (KBr) ν (cm^{-1}): 3015, 1218, 1150; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.43–4.18 (m, 8H), 3.53–3.42 (m, 6H), 3.33 (q, *J* = 7.4 & 14.8 Hz, 2H), 1.38 (t, *J* = 7.4 Hz, 3H); ^{13}C (100 MHz, $CDCl_3$): δ 198.1, 187.6, 186.5, 50.3, 48.0, 45.0, 43.5, 31.5, 13.6; ESI-MS: *m/z* 354 ($M+H^+$); Elemental analysis (%) for $C_{11}H_{19}N_3S_5$: Calcd.: C, 37.36; H, 5.42; N, 11.88; Found, C, 37.45; H, 5.75; N, 11.70.

4-(Butylthiocarbonothioyl)piperazine-1-carbothioic dimethylcarbamothioic thioanhydride (27)

The title compound (**27**) was synthesized from sodium 4-(butylthiocarbonothioyl)piperazine-1-carbodithioate (**24c**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 76% yield as yellow solid. mp: 85-87 °C; IR (KBr) ν (cm^{-1}): 3022, 1220, 1129; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.34–4.18 (m, 8H), 3.53–3.42 (m, 6H), 3.33 (t, *J* = 7.4 Hz, 2H), 1.75–1.67 (m, 2H), 1.51–1.42 (m, 2H), 0.96 (t, *J* = 7.3 Hz, 3H); ^{13}C (100 MHz, $CDCl_3$): δ 198.4, 187.6, 50.4, 45.0, 43.5, 37.0, 30.5, 22.1, 13.6; ESI-MS: *m/z* 382 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_{13}H_{23}N_3S_5 + H^+$ ($M+H^+$): 382.0568. Found: 382.0567. Elemental analysis (%) for $C_{13}H_{23}N_3S_5$: Calcd.: C, 40.91; H, 6.07; N, 11.01; Found, C, 40.70; H, 6.30; N, 11.13.

4-((3-cyanopropylthio)carbonothioyl)piperazine-1-carbothioic dimethylcarbamothioic thioanhydride (28)

The title compound (**28**) was synthesized from sodium 4-((3-cyanopropylthio)carbonothioyl)piperazine-1-carbodithioate (**24d**; 2 equiv.) and

dimethylcarbamothioic chloride (1 equiv.) in 69% yield as yellow solid. mp: 91-93 °C; IR (KBr) ν (cm^{-1}): 3020, 2231, 1216; ^1H NMR (400 MHz, $CDCl_3$): δ 4.37–4.12 (m, 8H), 3.54–3.42 (m, 8H), 2.51 (t, J = 7.2 Hz, 2H), 2.12 (q, J = 7.1 & 14.2 Hz, 2H); ^{13}C (100 MHz, $CDCl_3$): δ 196.6, 187.6, 187.3, 51.2, 50.1, 45.0, 43.5, 35.0, 28.8, 16.3; ESI-MS: *m/z* 393 ($M+H^+$); Elemental analysis (%) for $C_{13}H_{20}N_4S_5$: Calcd.: C, 39.77; H, 5.13; N, 14.27; Found, C, 39.51; H, 5.41; N, 14.12.

4-(Benzylthiocarbonothioyl)piperazine-1-carbothioic dimethylcarbamothioic thioanhydride (29)

The title compound (**29**) was synthesized from sodium 4-(benzylthiocarbonothioyl)piperazine-1-carbodithioate (**24e**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 80% yield as yellow solid. mp: 83-85 °C; IR (KBr) ν (cm^{-1}): 3410, 1639, 1442, 1216; ^1H NMR (400 MHz, $CDCl_3$): δ 7.40 (d, J = 7.5 Hz, 2H), 7.36–7.29 (m, 3H), 4.60 (s, 2H), 4.35–4.13 (m, 8H), 3.54–3.53 (m, 3H), 3.45–3.42 (m, 3H); ^{13}C (100 MHz, $CDCl_3$): δ 197.4, 187.6, 186.5, 135.5, 129.3, 128.6, 127.7, 50.1, 45.0, 43.5, 42.1; ESI-MS: *m/z* 416 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_{16}H_{21}N_3S_5$ + H^+ ($M+H^+$): 416.0412. Found: 416.0412. Elemental analysis (%) for $C_{16}H_{21}N_3S_5$: Calcd.: C, 46.23; H, 5.09; N, 10.11; Found, C, 46.01; H, 5.28; N, 10.25.

4-(Butylcarbamothioyl)piperazine-1-carbothioic dimethylcarbamothioic thioanhydride (33)

The title compound (**33**) was synthesized from sodium 4-(butylcarbamothioyl)piperazine-1-carbodithioate (**32a**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 76% yield as yellow solid. mp: 108-110 °C; IR (KBr) ν (cm^{-1}): 3018, 1216, 1192; ^1H NMR (400 MHz, $CDCl_3$): δ 5.48 (s, 1H), 4.35–4.08 (m, 8H), 3.71–3.66 (m, 2H), 3.53 (s, 3H), 3.43 (s, 3H), 1.68–1.60 (m, 2H), 1.46–1.36 (m, 2H), 0.97 (t, J = 7.3 Hz, 3H); ^{13}C (100 MHz, $CDCl_3$): δ 187.4, 186.5, 181.9, 50.6, 50.0, 46.1, 45.0, 43.6, 31.3, 20.1, 13.8; ESI-MS: *m/z* 365 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_{13}H_{24}N_4S_4$ + H^+ ($M+H^+$): 365.0957. Found: 365.0948. Elemental analysis (%) for $C_{13}H_{24}N_4S_4$: Calcd.: C, 42.82; H, 6.63; N, 15.37; Found, C, 42.59; H, 6.88; N, 15.09.

Dimethylcarbamothioic 4-(phenylcarbamothioyl)piperazine-1-carbothioic thioanhydride (34)

The title compound (**34**) was synthesized from sodium 4-(phenylcarbamothioyl)piperazine-1-carbodithioate (**32b**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 83% yield as yellow solid. mp: 82-84 °C; IR (KBr) ν (cm^{-1}): 3410, 1490, 1216, 1194, 1127; ^{1}H NMR (400 MHz, $CDCl_3$+DMSO-d_6): δ 8.83 (s, 1H), 7.14–7.13 (m, 4H), 6.99 (bs, 1H), 4.15–3.92 (m, 8H), 3.32–3.23 (m, 6H); ^{13}C (100 MHz, $CDCl_3$+DMSO-d_6): δ 192.1, 191.1, 187.1, 145.3, 133.1, 130.7, 130.1, 55.8, 55.2, 51.9, 49.7, 48.4; ESI-MS: *m/z* 385 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_{15}H_{20}N_4S_4$ + H^+ ($M+H^+$): 385.0644. Found: 385.0635. Elemental analysis (%) for $C_{15}H_{20}N_4S_4$: Calcd.: C, 46.84; H, 5.24; N, 14.57; Found, C, 46.50; H, 5.49; N, 14.40.

4-(Benzylcarbamothioyl)piperazine-1-carbothioic dimethylcarbamothioic thioanhydride (35)

The title compound (**35**) was synthesized from sodium 4-(benzylcarbamothioyl)piperazine-1-carbodithioate (**32c**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 83% yield as yellow solid. mp: 218-220 °C; IR (KBr) ν (cm^{-1}): 3410, 1639, 1402, 1216, 1185; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35–7.27 (m, 4H), 6.20–6.07 (m, 1H), 4.87–4.86 (m, 2H), 4.27–4.08 (m, 8H), 3.50–3.39 (m, 6H); ^{13}C (100 MHz, $CDCl_3$): δ 187.4, 186.5, 182.0, 137.6, 128.8, 128.0, 50.2, 46.3, 45.0, 43.6, 30.9; ESI-MS: *m/z* 399 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_{16}H_{22}N_4S_4$ + H^+ ($M+H^+$): 399.0800. Found: 399.0791. Elemental analysis (%) for $C_{16}H_{22}N_4S_4$: Calcd.: C, 48.21; H, 5.56; N, 14.05; Found, C, 48.02; H, 5.85; N, 14.29.

Dimethylcarbamothioic 4-(phenethylcarbamothioyl)piperazine-1-carbothioic thioanhydride (36)

The title compound (**36**) was synthesized from sodium 4-(phenethylcarbamothioyl)piperazine-1-carbodithioate (**32d**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 70% yield as yellow solid. mp: 85-87 °C; IR (KBr) ν (cm^{-1}): 3410, 1639, 1402, 1216, 1185; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36–7.31 (m, 2H), 7.26–7.22 (m, 3H), 5.54–5.53 (m, 1H), 4.38–3.93 (m, 10H), 3.52–3.41 (m, 6H),

3.00–2.96 (m, 2H); ^{13}C (100 MHz, $CDCl_3$): δ 187.4, 186.5, 181.8, 138.7, 128.7, 126.7, 50.6, 50.0, 47.0, 45.9, 45.0, 43.7, 35.1; ESI-MS: *m/z* 413 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_{17}H_{24}N_4S_4 + H^+$ ($M+H^+$): 413.0957. Found: 413.0946. Elemental analysis (%) for $C_{17}H_{24}N_4S_4$: Calcd.: C, 49.48; H, 5.86; N, 13.58; Found, C, 49.32; H, 6.11; N, 13.31.

4-(Cyclohexylcarbamothioyl)piperazine-1-carbothioic dimethylcarbamothioic thioanhydride (37)

The title compound (**37**) was synthesized from sodium 4-(cyclohexylcarbamothioyl)piperazine-1-carbodithioate (**32e**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 70% yield as yellow solid. mp: 238-240 °C; IR (KBr) ν (cm^{-1}): 3019, 1216, 1190; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.32–5.30 (m, 1H), 4.36–4.06 (m, 8H), 3.68–3.60 (m, 1H), 3.53–3.42 (m, 6H), 2.14–2.09 (m, 2H), 1.76–1.65 (m, 3H), 1.48–1.38 (m, 2H), 1.15–1.24 (m, 3H); ^{13}C (100 MHz, $CDCl_3$): δ 187.5, 186.5, 180.7, 54.6, 50.6, 49.9, 45.8, 45.0, 43.6, 33.0, 25.5, 24.9; ESI-MS: *m/z* 391 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_{15}H_{26}N_4S_4 + H^+$ ($M+H^+$): 391.1113. Found: 391.1123. Elemental analysis (%) for $C_{15}H_{26}N_4S_4$: Calcd.: C, 46.12; H, 6.71; N, 14.34; Found, C, 46.20; H, 6.99; N, 14.06.

4-(Cyclohexylcarbamoyl)piperazine-1-carbothioic dimethylcarbamothioic thioanhydride (38)

The title compound (**38**) was synthesized from sodium 4-(cyclohexylcarbamoyl)piperazine-1-carbodithioate (**32f**; 2 equiv.) and dimethylcarbamothioic chloride (1 equiv.) in 73% yield as yellow solid. mp: 109-111 °C; IR (KBr) ν (cm^{-1}): 3020, 1215, 1150; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.30 (s, 3H), 4.14–4.02 (m, 2H), 3.67–3.60 (m, 4H), 3.54–3.53 (m, 3H), 3.45–3.43 (m, 3H), 1.98–1.96 (m, 2H), 1.74–1.71 (m, 2H), 1.64 (s, 1H), 1.43–1.35 (m, 2H), 1.30 (s, 1H), 1.27–1.08 (m, 3H); ^{13}C (100 MHz, $CDCl_3$): δ 187.5, 186.7, 156.5, 51.6, 50.4, 49.6, 44.9, 43.7, 42.6, 33.9, 25.6, 25.0; ESI-MS: *m/z* 418 ($M+H^+$); HRMS (ESI): *m/z* calculated for $C_{15}H_{26}N_4OS_3 + H^+$ ($M+H^+$): 375.1341. Found: 375.1348. Elemental analysis (%) for $C_{15}H_{26}N_4OS_3$: Calcd.: C, 48.10; H, 7.00; N, 14.96; Found, C, 48.22; H, 7.31; N, 14.64.

Biological Materials and Methods:

Anti-*Trichomonas* assay

Metronidazole-susceptible *T. vaginalis* strain was a clinical isolate obtained from the laboratory of Divya Singh [37] and metronidazole-resistant strain of *T. vaginalis* (CDC085 [ATCC 50143]) was procured from American Type Culture Collection. *In vitro* drug susceptibility of *Trichomonas vaginalis* was assayed as detailed earlier.[16] Briefly, parasites were incubated at 37°C in the presence of test compounds or MTZ, serially diluted from a stock-solution in DMSO using the culture medium, in 48-well culture plates. 0.05% DMSO in culture media (the highest concentration of DMSO in test wells) was used as vehicle in control wells. Cell viability was checked after 48 h by trypan blue exclusion assay. The minimum concentration of the test agent at which all cells were found dead was considered as its MIC. MTZ (the most widely used drug against *Trichomonas vaginalis*) was procured from Sigma-Aldrich, and used as reference standard. All experiments were repeated three times (Table 1).

Antifungal assay

The MIC of compounds were determined by broth micro-dilution technique as per the guidelines of National Committee for Clinical Laboratory Standards using RPMI 1640 media buffered with MOPS [3-(N-Morpholino)propanesulfonic acid]. Starting inoculums of test culture was 1–5 x 10^3 CFU/mL. Micro titer plates were incubated at 35 °C. MICs were recorded after 48h of incubation. All experiments were repeated three times.[22] (Table 2).

Cytotoxicity of compounds toward human cervical (*HeLa*) cells

The cytotoxic effect of test compounds were evaluated in an in vitro model of cervicovaginal epithelium (HeLa) cells, using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay.[24] Cells seeded in 96 well plates were incubated in culture medium (DMEM with 10% fetal calf serum) for 24 h at 37°C in a 5% CO2–95% air atmosphere. After 24 h, the culture medium was replaced with fresh medium containing serial dilutions of test compounds starting with 1000 μg/ml in experimental

wells and 0.05% DMSO in culture medium in control wells. After incubation for another 24 h, 5 µL of 5 mg/mL MTT solution in PBS [pH 7.4] was added to each well. The formazan crystals formed inside the viable cells were solubilized in DMSO, and the optical density at 540 nm (OD540) was recorded in a microplate reader (Microquant; BioTek). All experiments were repeated three times. (Table 1)

Qualitative estimation of inhibition of free sulfhydryl groups on *Trichomonas vaginalis*

The effect of test compound on *Trichomonas* free sulfhydryl groups was examined and imaged by a method published earlier with slight modification [38] using a fluorescence microscope, after labelling with the fluorometric thiol detector using a thiol –detection assay kit (Cayman). *Trichomonas vaginalis* was treated with the vehicle or the test compound at MIC and incubated for 24 h at 37°C. After incubation, trichomonads were pelleted at 700 xg for 10 min at 4°C and washed 2-3 times with PBS. Thereafter 50 µL fluorometric thiol detector (pre-diluted 100x with dilution buffer), was added and incubated in the dark. A drop of this sample was taken on a microscope slide, covered with a coverslip and imaged on a Nikon Eclipse 80i microscope equipped with epifluoresence illumination, using the UV-1A filter. Exposure times were the same for all samples.

Subcutaneous abscess assay for measuring anti-Trichomonal efficacy of test compounds in the mouse model

The subcutaneous abscess assay of Krieger et. al. [39] was used. Briefly, the parasites (*T. vaginalis*) were cultured under partial anaerobic condition in TYM medium and on attaining concentration of approximately $2x10^6$ cells/mL (in ~48 hrs) trichomonads were harvested from the culture by centrifugation at 250 xg for 10 min and then re-suspended in sterile saline. Six week old mice were inoculated subcutaneously with *T. vaginalis* (50 µL of 2×10^6 organisms per mL) into the left hind flank. Control animals were injected with sterile saline only. Five groups were used for each experiment (n=3). The abscess / lesion formation was determined by palpation 7 days after injection, and measured daily thereafter. Fine needle biopsy specimens

were taken from the lesion and examined microscopically to ensure infection**.** Infected animals were then treated with compounds (orally) with a dose of 50 mg/kg and 100 mg/kg for 7 days and abscess size measured longitudinally and the area calculated as πr^2. MTZ was used as positive control. The assay was approved by the Institutional Animal Ethics Committee.[28]

Docking study

The sequence of Trichomonas vaginalis cysteine synthase (TvCS) was retrieved from Uniprot (A2GMG5). Since crystal structure of TvCS is not available, therefore a homology model was constructed using crystal structure of cysteine synthase from Escherichia coli (PDB ID-2BHS) [40] as template with the help of MODELLER package. [41] All docking studies were carried using AUTODOCK4.2 Package.51 For molecular visualization and structure manipulation Chimera was used. [42]

***In vivo* pharmacokinetic assay**

The pharmacokinetic studies of compound **20** was carried out in young and healthy male *Sprague Dawley* rats weighing 250±25 g obtained from laboratory animal division, CSIR-CDRI, Lucknow. The animals were housed in plastic cages in standard laboratory conditions with a regular 12 hr day-night cycle. Standard pelleted laboratory chow (Goldmohar Laboratory Animal Feed, Lipton India Ltd, Chandigarh, India) and water were allowed *ad libitum.* The rats were acclimatized to this environment for at least five days before conducting the experiment. The oral dose pharmacokinetic study was conducted in overnight fasted (12-16 hr) rats (n = 4 per time point). All experiments, euthanasia and disposal of carcasses were carried out as per the guidelines of Local Ethics Committee for animal experimentation. Suspension formulations containing 12.5 mg/mL of compound was prepared separately by triturating the compound, gum acacia (1% w/v) and water (drop wise addition) in mortar and pestle. A single 50 mg/kg oral dose was given to conscious rats using rat feeding needle. Blood samples were withdrawn at various predefined times up to 24 hr post dose. Serum samples were harvested and stored at -80 °C until analysis. A Shimadzu UFLC pump (LC-20AD) with online degasser (DGU-20A3), an auto-sampler (SIL-HTc) with a temperature-controlled peltier-tray and a triple quadrupole API 4000 mass spectrometer (Applied Biosystems, Toronto, Canada) was used for analysis. Chromatographic separation was made on a Discovery HS C-18 column (5 µm, 50x4.6 mm id)

preceded with a guard column (5 µm, 20 x 4.0 mm, id) packed with the same material with mobile phase [acetonitrile: aqueous ammonium acetate buffer (0.01M; pH, 4.5) (80:20, %v/v)] pumped at a flow rate of 0.6 mL/min under isocratic condition. The mobile phase was degassed by ultra sonication for 15 min before use. LC-MS/MS system was equilibrated for approximately 20 min before commencement of analysis. Total analysis time was 3 min per sample. The mass spectral analysis was performed in positive ionization mode at 5500 V using multiple reaction monitoring technique to monitor the transitions *m/z* 328.3→ *m/z* 206.9 for compound **20** and *m/z* 180.1 →*m/z* 138.1 for phenacetin (internal standard). Data acquisition and quantitation were performed using analyst software (version 1.4.2; AB Sciex, Toronto, Canada). The method utilizes 50 µL of serum. For sample clean up protein precipitation was used. The method showed linearity over the range of 1- 200 ng/mL with recovery of >50% and acceptable accuracy and precision [43] [FDA, Guidance for Industry: Bioanalytical Method Validation].

Supporting Information:

^{1}H NMR, ^{13}C NMR spectra of the compounds, HRMS spectra and supplementary were available free of charge via internet at ……..

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Abbreviations:

STDs, sexually transmitted disease; TV, *Trichomonas vaginalis*; DTC, dithiocarbamate; SAR, structure activity relationship; MTZ, Metronidazole; NI, nitroimidazoles; *HeLa*, epithelium cells; 3D-QSAR, 3D-quantitative structure–activity relationship; SAR, structure–activity relationship; MIC, minimum inhibitory concentration; IC_{50}, half maximal inhibitory concentration; CoMFA, comparative molecular field analysis; PLS, partial least squares; LDH, lactate dehydrogenase; DMEM, dulbecco's modified eagle's medium; TLC, thin layer chromatography.

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Table captions:

Table 1: Anti-Trichomonas activity of synthesized compounds (**3-21**, **25-29** and **33-38**)[a]

Table 2: Antifungal activity of synthesized compounds (**2-20** and **32-42**)[a]

Table 3: Pharmacokinetic parameters of compound **20** after single 50 mg/kg administration in male *Sprague Dawley* rats.[a]

Figure captions:

Figure 1: Previous lab work lead molecules (**a-d**), structural modifications led to designed prototype (**e**).

Figure 2: SAR for anti-Trichomonas activity

Figure 3: Fluorescent labeling of Trichomonas sulfhydryls with thiol detector: (i) Fluorescence image, (ii) Phase contrast image, (iii) Merged image.

Figure 4: Docked complex of most active compound **20** found in this study. Compound **20** is shown in magenta color, protein residues are depicted in cyan color and H-bond are shown as black dashed lines.

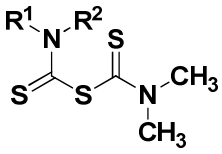
Figure 5. Abscess size in mice at days 1, 2, 5 and 7 (autopsy day). Mean ± SE of three independent experiments. Significant difference from the diseased /untreated indicated as ***P < 0.001.

Scheme captions:

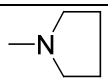
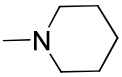
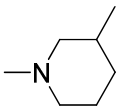
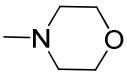
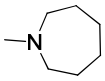
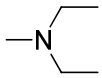
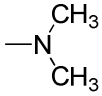
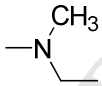
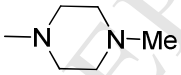
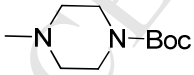
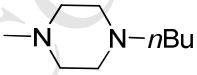
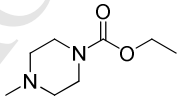
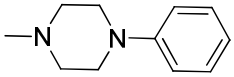
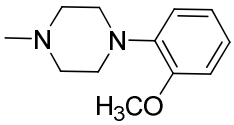
Scheme 1. Synthesis of compounds **3-21**, **25-29**, **33-38**.

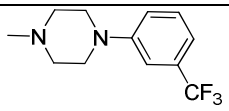
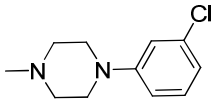
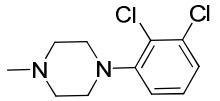
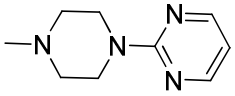
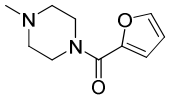
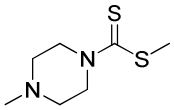
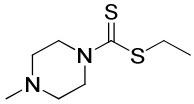
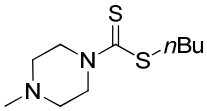
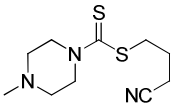
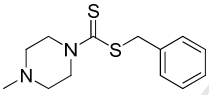
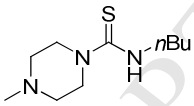
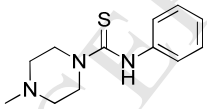
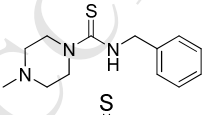
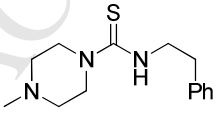
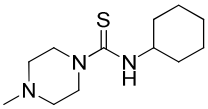
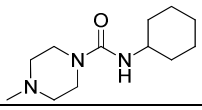
Reagents and Reaction Conditions: a) Carbon disulfide, aq. NaOH, EtOAc, 0–5 °C, 30 min; b) dimethylcarbamothioic chloride, CH_3CN, 0–5 °C, 1-2 hr; c) Substituted alkyl halides, water:acetone (10:1), 15-20 ^oC, 30-40 min; d) TFA, DCM, aq. $NaHCO_3$, 0–5 °C, 5-6 hr; e) Substituted isothiocyanate/thiocyanate, ethanol, rt, 3–4 h;

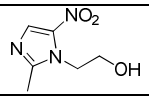
Table 1: Anti-*Trichomonas* activity of synthesized compounds (**3-21**, **25-29** and **33-38**)[a]



(3-21, 25-29, 33-38)

Compound	-NR^1R^2	MTZ susceptible strain MIC[b] (µM)	MTZ resistant strain[c] MIC (µM)	*HeLa*[d] IC_{50}[e] (µM)	Selectivity index (IC_{50}/MIC) MTZ susceptible strain	Selectivity index (IC_{50}/MIC) MTZ resistant strain
3		26.70	106.83	3094.01	115	29
4		25.20	100.80	>5000	>200	>50
5		47.70	95.41	2900.76	60	30
6		100.00	400.00	4000.00	40	10
7		95.41	190.83	>5000	>52	>26
8		52.96	105.93	>5000	>94	>47
9		60.09	240.38	>5000	>83	>20
10		112.61	225.22	2067.56	18	9
11		47.52	95.05	1220.53	26	12
12		17.85	71.42	1308.57	73	18
13		20.42	81.69	4098.03	200	50
14		77.88	155.76	529.59	6	3
15		38.34	76.68	1607.36	42	21
16		280.89	702.24	4334.26	15	6

17		63.45	126.90	2758.88	43	21
18		138.88	277.77	>5000	36	18
19		63.45	126.90	1593.90	25	12
20		4.77	38.22	>5000	1048	131
21		18.22	145.77	2379.00	130	16
25		294.11	735.29	>5000	>17	>7
26		141.24	282.48	4483.05	31	15
27		261.78	654.45	>5000	>19	>7
28		63.61	127.22	>5000	>79	>39
29		60.09	120.19	>5000	>83	>41
33		68.49	136.98	2835.61	41	20
34		8.10	32.46	>5000	>617	>154
35		125.31	250.62	>5000	>40	>20
36		60.53	242.13	>5000	>83	>20
37		31.96	63.93	>5000	>161	>79
38		133.33	266.66	>5000	>37	>18

MTZ[f]		18.24	365.49	>5000	>277	>13

[a] all the experiments were carried out in triplicate; [b] minimum inhibitory concentration; [c] ATCC 50143 strain; [d] human cervical cell line; [e] half maximal inhibitory concentration; [f] Metronidazole.

Table 2: Antifungal activity of synthesized compounds (**2-20** and **32-42**)[a]

Comp.	Antifungal activity (MIC,[b] µM)									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
3	213.67	53.41	53.41	106.83	53.41	26.70	53.41	53.41	53.41	106.83
4	201.61	100.80	100.80	100.80	50.40	25.20	50.40	50.40	50.40	100.80
5	47.70	23.85	23.85	23.85	95.41	95.41	47.70	95.41	47.70	23.85
6	200.00	100.00	100.00	200.00	25.00	50.00	100.00	100.00	50.00	200.00
7	47.70	23.85	23.85	23.85	23.85	11.90	23.85	11.90	47.70	23.85
8	105.93	26.48	26.48	52.96	>211.86	>211.86	52.96	>211.86	105.93	26.48
9	240.38	60.09	60.09	120.19	120.19	120.19	60.09	60.09	120.19	120.19
10	225.22	56.30	56.30	112.61	112.61	112.61	28.15	112.61	56.30	112.61
11	190.11	95.05	95.05	95.05	190.11	95.05	47.52	190.11	95.05	190.11
12	142.85	142.85	142.85	142.85	142.85	142.85	71.42	142.85	142.85	142.85
13	163.39	163.39	163.39	163.39	163.39	163.39	81.69	163.39	81.69	163.39
14	155.76	77.88	77.88	155.76	>155.76	155.76	38.94	155.76	155.76	155.76
15	76.68	76.68	76.68	38.34	>153.37	>153.37	>153.37	>153.37	>153.37	76.68
16	>140.44	140.44	140.44	>140.44	>140.44	>140.44	70.22	>140.44	140.44	70.22
17	63.45	15.86	15.86	31.72	>126.90	>126.90	>126.90	>126.90	63.45	15.86
18	138.66	34.66	34.66	34.66	>138.66	>138.66	>138.66	>138.66	69.33	8.66
19	126.90	15.86	15.86	31.72	>126.90	>126.90	>126.90	>126.90	63.45	31.72
20	152.90	152.90	152.90	152.90	152.90	152.90	38.22	152.90	76.45	152.90
21	145.77	72.88	72.88	145.77	145.77	145.77	72.88	145.77	72.88	145.77
25	>147.05	147.05	147.05	>147.05	>147.05	>147.05	>147.05	>147.05	>147.05	36.76

26	141.01	35.25	35.25	17.62	141.01	141.01	70.50	>141.01	70.50	8.81
27	65.34	32.67	32.67	16.33	>130.68	130.68	32.67	130.68	130.68	8.16
28	>127.22	127.22	127.22	>127.22	>127.22	>127.22	>127.22	>127.22	>127.22	>127.22
29	60.09	60.09	60.09	30.04	>120.19	120.19	30.04	120.19	120.19	7.50
33	>136.98	136.98	136.98	136.98	>136.98	>136.98	>136.98	>136.98	>136.98	136.98
34	>129.87	129.87	129.87	>129.87	>129.87	>129.87	>129.87	>129.87	>129.87	>129.87
35	>125.31	125.31	125.31	>125.31	>125.31	>125.31	>125.31	>125.31	>125.31	>125.31
36	>121.06	>121.06	>121.06	>121.06	>121.06	>121.06	>121.06	>121.06	>121.06	>121.06
37	>127.87	127.87	127.87	>127.87	>127.87	>127.87	>127.87	>127.87	>127.87	127.87
38	>133.33	133.33	133.33	>133.33	>133.33	>133.33	>133.33	>133.33	>133.33	133.33
Fluc.[c]	6.53	6.53	>104.57	3.26	3.26	0.39	1.63	13.07	26.14	6.53

*[a] all the experiments were carried out in triplicate; [b] minimum inhibitory concentration;[c] Flucanazole; **1**. Cryptococcus neoformans; **2.** Sporothrix schenckii; **3.** Trichophyton mentagrophytes; **4.** Candida albicans Patient isolate; **5.** Candida albicans ATCC-10231; **6.** Candida albicans ATCC-14053; **7.** Candida albicans ATCC-66027; **8.** Candida glabrata ATCC-2001; **9.** Candida albicans MTCC-183; **10.** Candida parapsilosis ATCC-22019.*

Table 3 : Pharmacokinetic parameters of compound **20** after single 50 mg/kg administration in male *Sprague Dawley* rats.[a]

Parameters	Compound 20
C_{max} (ng/mL)	47.1±2.8
T_{max} (hr)	2.0±0.0
AUC_{last} (ng*hr/mL)	140.5±4.2
$t_{1/2}$ (hr)	2.6 ±0.0[b]
Cl (L/hr/kg)	4.9±0.2
V_{ss} (L/kg)	12.6±0.6

[a] Each value represent the average of four rats; [b] MRT(mean residence time)

Abbreviations: AUC_{last} = area under the concentration-time curve up to last observation, C_{max} = peak serum concentration, t_{max} = time to C_{max}, V_{ss} = steady-state volume of distribution, Cl = clearance, $T_{1/2}$= elimination half-life.

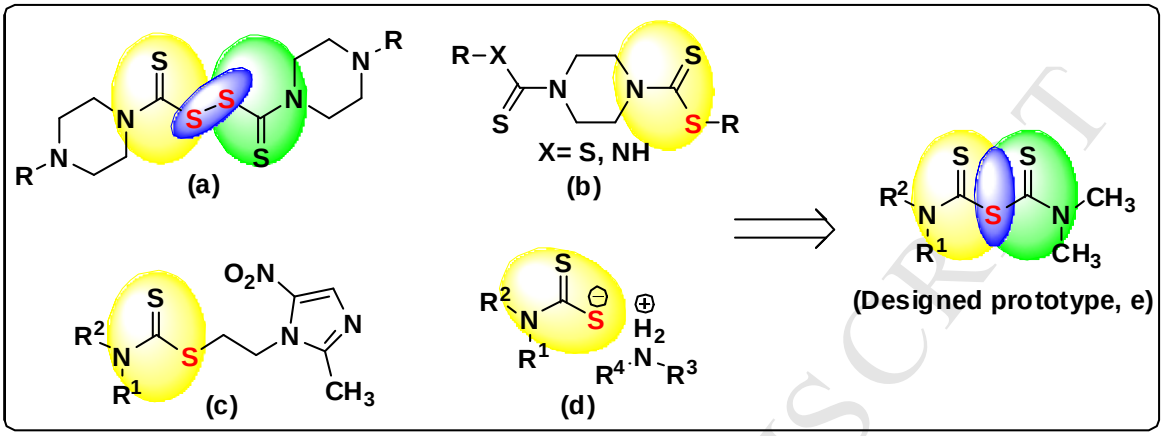
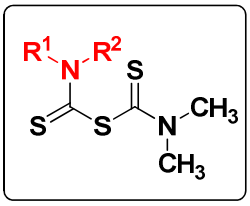


Figure 1: Previous lab work lead molecules (**a**-**d**), structural modifications led to designed prototype (**e**).



$-NR^1R^2$ = <u>Substitution order for good anti-Trichomonas activity</u>:

Piperazine substitutions>Piperazine-Thiourea hybrids >cyclic amines>Piperazine-DTC hybrids

<u>Substitution order for good anti-fungal activity</u>:

Piperazine-DTC hybrids>Piperazine substitutions>cyclic amines>Piperazine-Thiourea hybrids

Figure 2: SAR for anti-Trichomonas activity

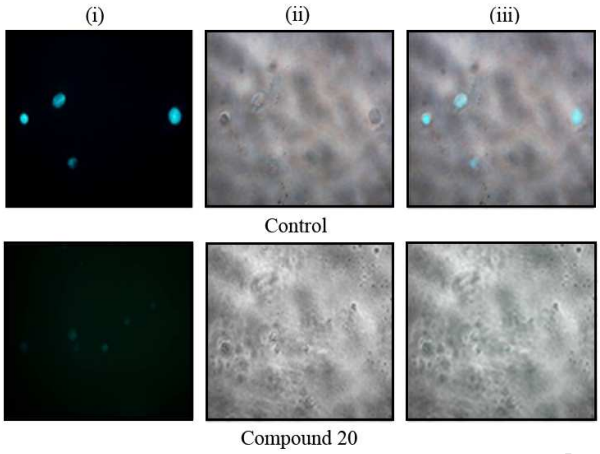


Figure 3. Fluorescent labeling of *Trichomonas* sulfhydryls with thiol detector: (i) Fluorescence image, (ii) Phase contrast image, (iii) Merged image.

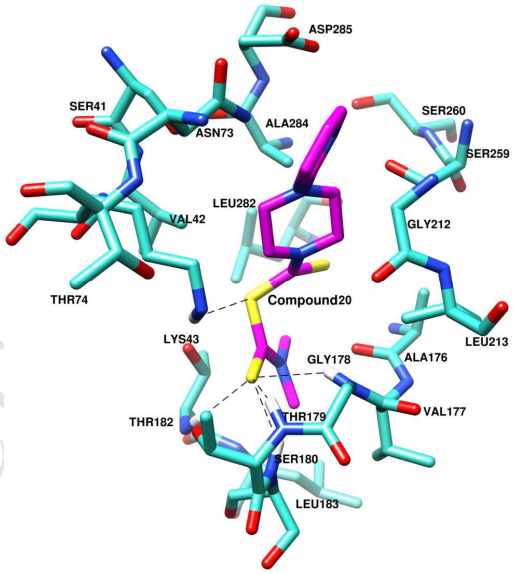


Figure 4: Docked complex of most active compound **20** found in this study. Compound **20** is shown in magenta color, protein residues are depicted in cyan color and H-bond are shown as black dashed lines.

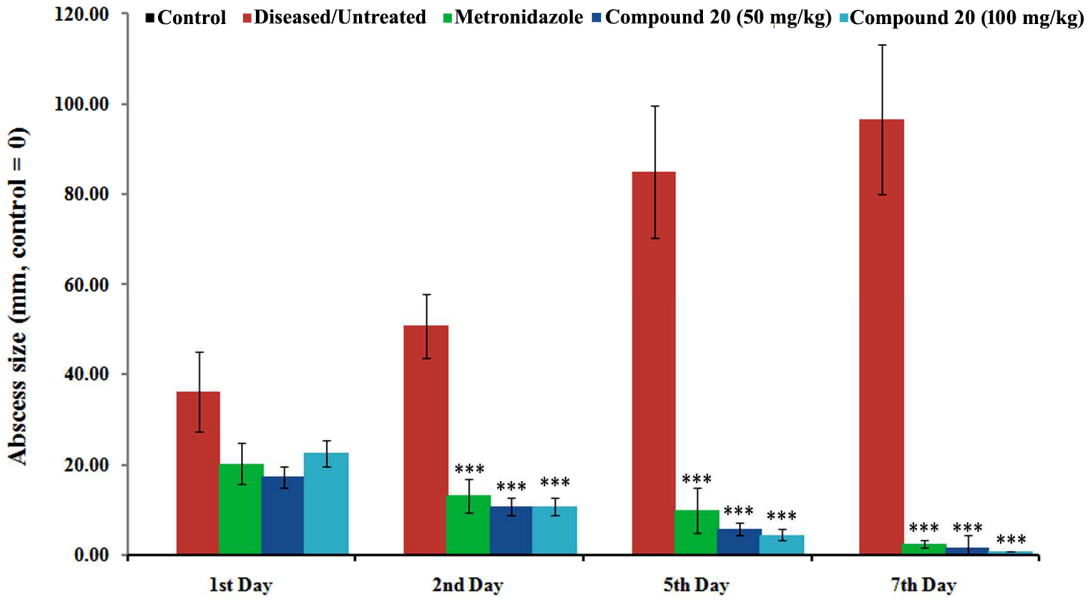
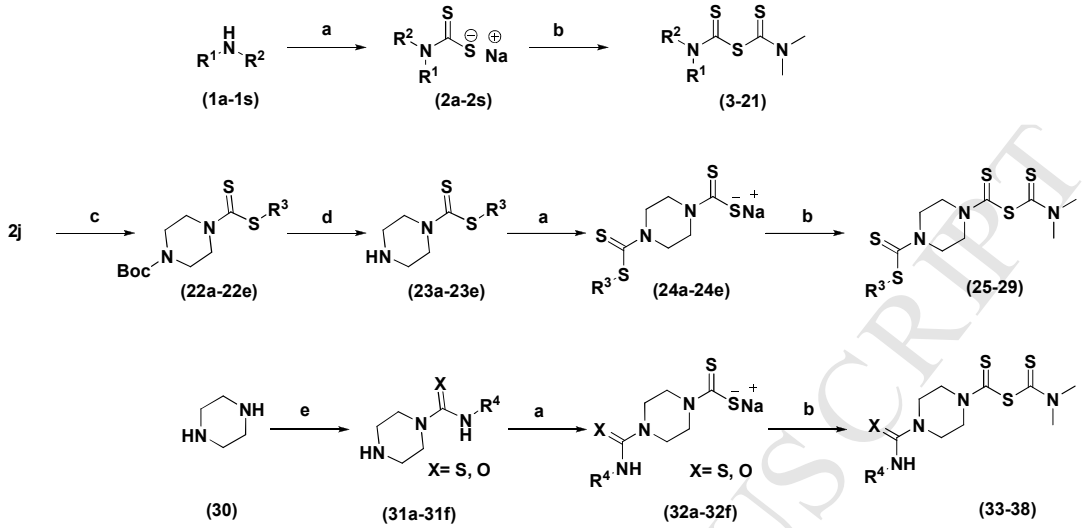


Figure 5. Abscess size in mice at days 1, 2, 5 and 7 (autopsy day). Mean ± SE of three independent experiments. Significant difference from the diseased /untreated indicated as ***P < 0.001.

Scheme 1:



-NR^1R^2: 1a, 2a, 3: pyrrolidine
1b, 2b, 4: piperidine
1c, 2c, 5: 3-methylpiperidine
1d, 2d, 6: morpholine
1e, 2e, 7: azepane
1f, 2f, 8: N,N'-diethyl
1g, 2g, 9: N,N'-dimethyl
1h, 2h,10: N,N'-ethyl(methyl)
1i, 2i, 11: 4-methylpiperazine
1j, 2j, 12: 4-(*tert*-Butoxycarbonyl)piperazine

-NR^1R^2: 1k, 2k, 13: 4-Butylpiperazine
1l, 2l, 14: 4-(Ethoxycarbonyl) piperazine
1m,2m,15:4-phenylpiperazine
1n, 2n,16: 4-(2-Methoxyphenyl)piperazine
1o, 2o,17: 4-(2-(Trifluoromethyl)phenyl)piperazine
1p, 2p,18: 4-(3-Chlorophenyl)piperazine
1q, 2q,19: 4-(2,3-Dichlorophenyl)piperazine
1r, 2r, 20: 4-(pyrimidin-2-yl)piperazine
1s, 2s,21: 4-(furan-2-carbonyl)piperazine

R^3: 23a, 24a, 25: methyl
23b, 24b, 26: ethyl
23c, 24c, 27: butyl
23d, 24d, 28: 3-cyanopropyl
23e, 24e, 29: benzyl

R^4: 31a, 32a, 33: butyl; X=S
31b, 32b, 34: phenyl; X=S
31c, 32c, 35: benzyl; X=S
31d, 32d, 36: phenethyl; X=S
31e, 32e, 37: cyclohexyl; X=S
31f, 32f, 38: cyclohexyl; X=O

Reagents and Reaction Conditions: a) Carbon disulfide, aq. NaOH, EtOAc, 0–5 °C, 30 min; b) dimethylcarbamothioic chloride, CH_3CN, 0–5 °C, 1-2 hr; c) Substituted alkyl halides, water:acetone (10:1), 15-20 ^oC, 30-40 min; d) TFA, DCM, aq. $NaHCO_3$, 0–5 °C, 5-6 hr; e) Substituted isothiocyanate/thiocyanate, ethanol, rt, 3–4 h;

1. Thirty new compounds comprising different structural variations were synthesized.
2. Evaluated for MTZ-susceptible, –resistant *Trichomoniasis*, and fungal activities.
3. Several compounds were found more active than MTZ against both the strains tested.
4. Compound **20** showed good *in vivo* activity and thiol inhibition on *Trichomonas*.
5. Most active compounds of the series exhibited moderate antimalarial activity.